CRISPR/Cas9 Targeted Genome Editing: Tips and Considerations

Dr. Mark I.R. Petalcorin
Associate Professor of Biochemistry
PAPRSB Institute of Health Sciences
Universiti of Brunei Darussalam

Outline

- Introduction and history of CRISPR/Cas9
- Use of CRISPR/Cas9 for genome editing
- Key considerations in experimental set up
- Issues relevant for CRISPR/Cas9 application

Some of the latest developments in the field including the tools and resources available to help design and implement CRISPR-based experiments

Focus mainly on reviewing strategies
for editing coding genes to uncover their function

Biological toolbox

- DNA Sequencing
- Restriction enzymes for cloning
- PCR to synthesize DNA
- Genome engineering to rewrite genes

NGS: DNA information deluge

Availability of sequencing data (Genotype)

Challenge: How to relate these data to phenotype

Forward Genetics vs Reverse Genetics

Genome editing/engineering

a process of making targeted modifications to the genome, its context (e.g. epigenetic marks) and its outputs (e.g. transcripts)

disruption, deletion, insertion, replacement at a locus in a genome

Researchers have gained the ability to achieve targeted genomic modifications with efficiency and ease combined with the rapidly increasing amount of information available from genomic sequencing efforts available as well as innovative nucleic acid synthesis and delivery systems.

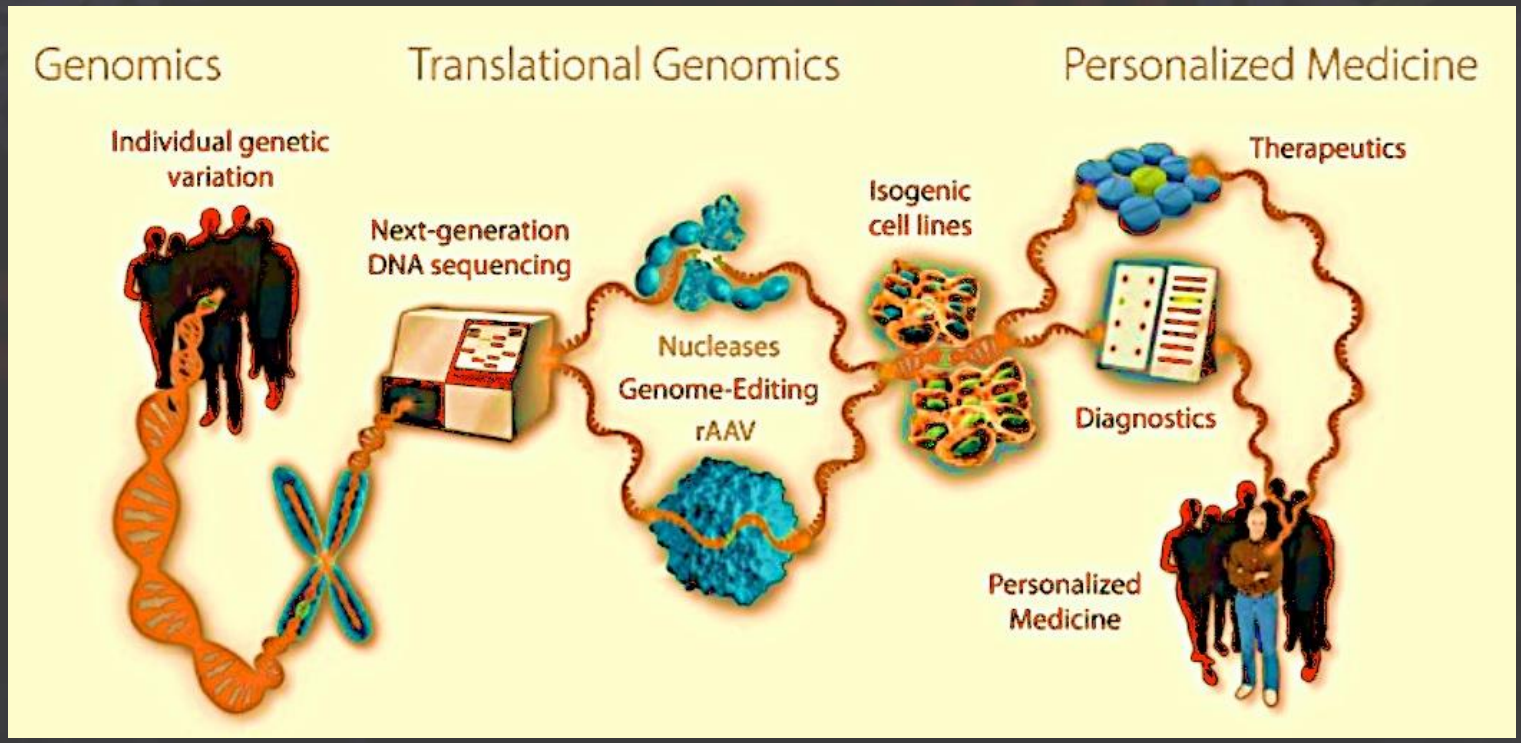
DNA information deluge

The opportunity:

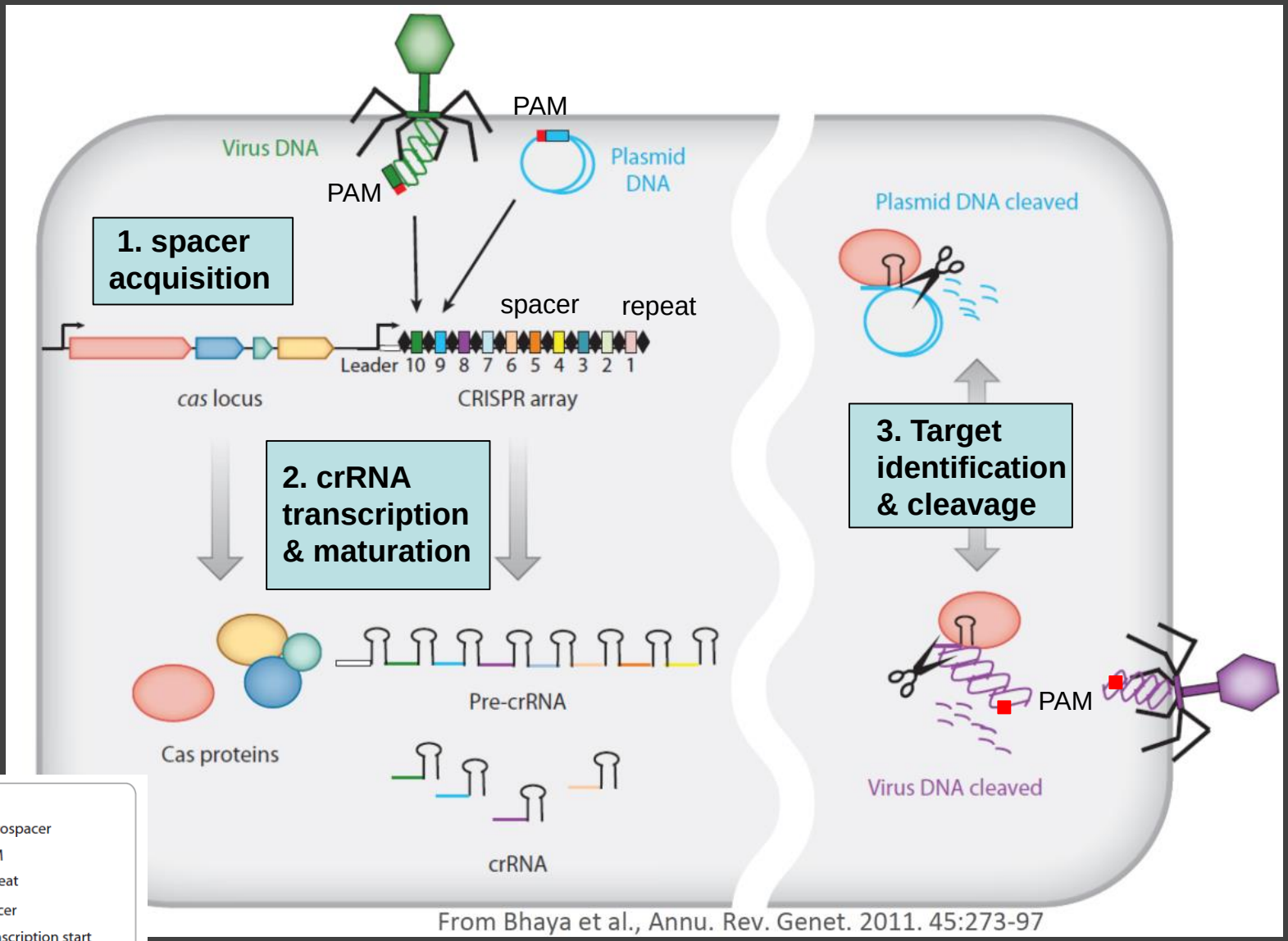
- translating genetic information into personalized medicines

Genome editing

- enables genomic data to be used in novel therapeutics and diagnostics
- increases efficiency of introducing targeted alterations into any specific gene in living cells



CRISPR system mediated adaptive immunity



From Bhaya et al., Annu. Rev. Genet. 2011. 45:273-97

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Cas proteins: CRISPR-Associated proteins

Classification of CRISPR system

CRISPR-Cas systems detected in 47% of all bacteria/archaeal genomes

Five system types based on sequence and structure of Cas protein

- Types I *cas3* 60% of total bacterial/archaeal genomes
- Type II *cas9* <5% of all bacterial genomes
- Type III *cas10* 34% of all archaeal, 25% in bacterial genomes
- Type IV rare <2% of overall CRISPR-Cas systems

- Type V *cpf1* rare <2% of overall CRISPR-Cas systems
- crRNA-guided surveillance complexes in Types I and III need multiple complex Cas subunits called Cascade or Cmr/Csm
- Type II requires only Cas9 and Type V requires only Cpf1

Briner and Barrangou 2016 CSHL press

Cas9 nuclease (formerly Csn1 or Csx12)

cleaves dsDNA (sequence specific)

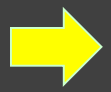
- RuvC-like nuclease domain at N-terminus
  - named after *E.coli* DNA repair protein
- HNH (or McrA-like) nuclease domain at the middle
  - named after histidine and asparagine residues

- Each of the domains cuts opposite DNA strand to generate DSB (Double-strand break)

CRISPR/Cas9 Type II system

Natural four components:

1. Cas9 nuclease
2. RNaseIII
3. crRNA (CRISPR RNA)
4. trans-activating tracrRNA

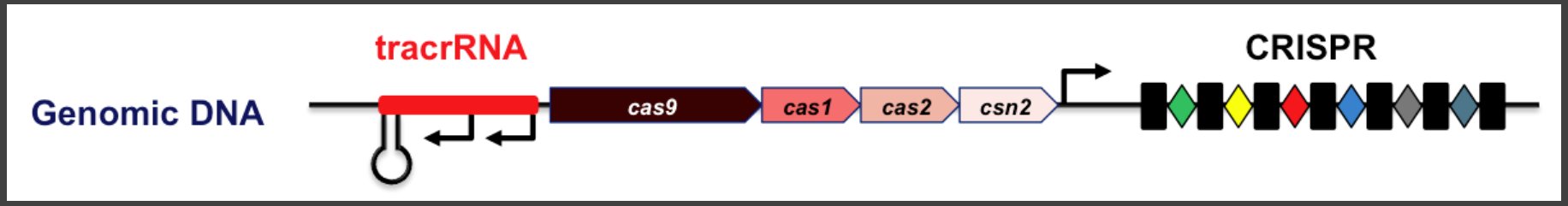


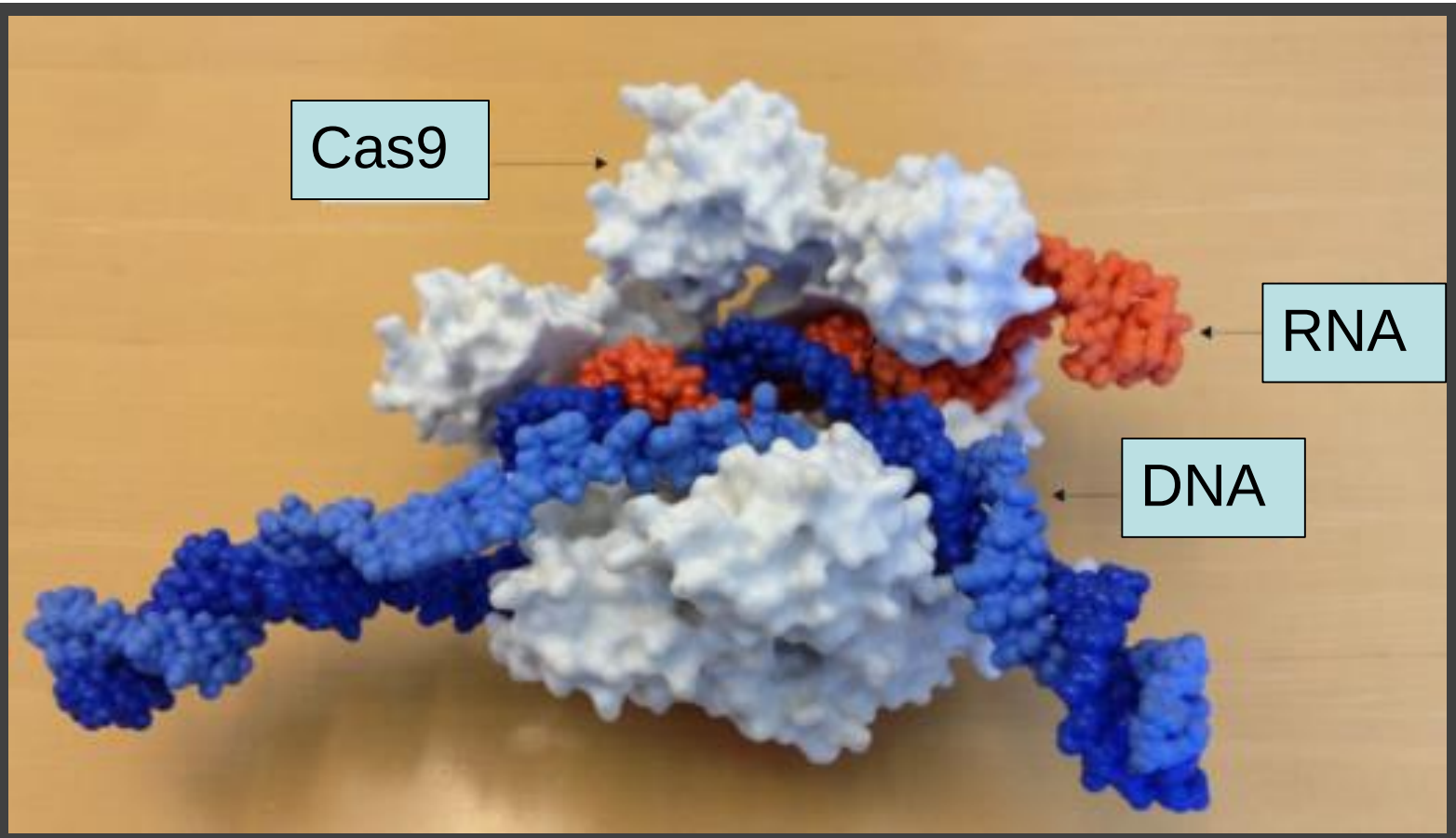
Synthetic two components:

1. Cas9 nuclease
2. sgRNA

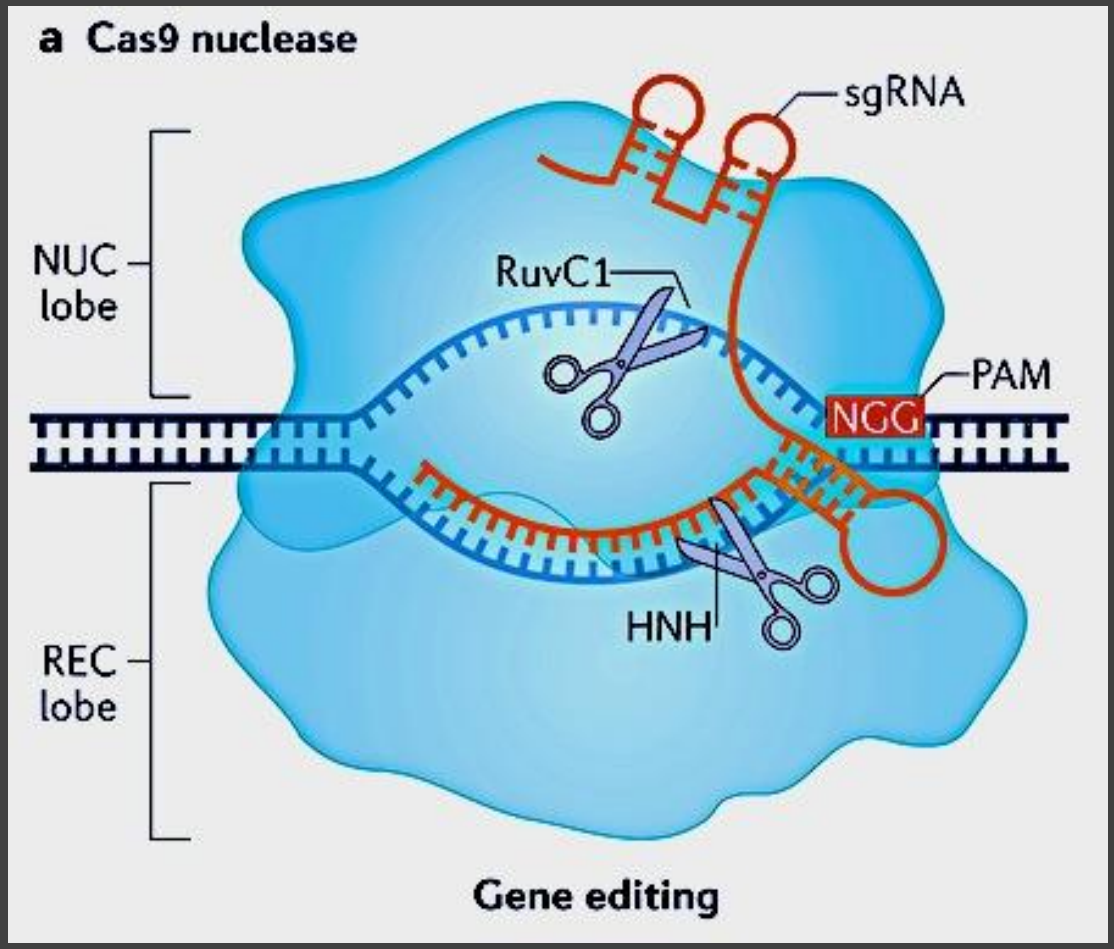
Cas9-based technology became a programmable genome editing tool by artificially combining crRNA:tracrRNA duplex as a single guide RNA (sgRNA).

The *Streptococcus pyogenes* Cas9 endonuclease



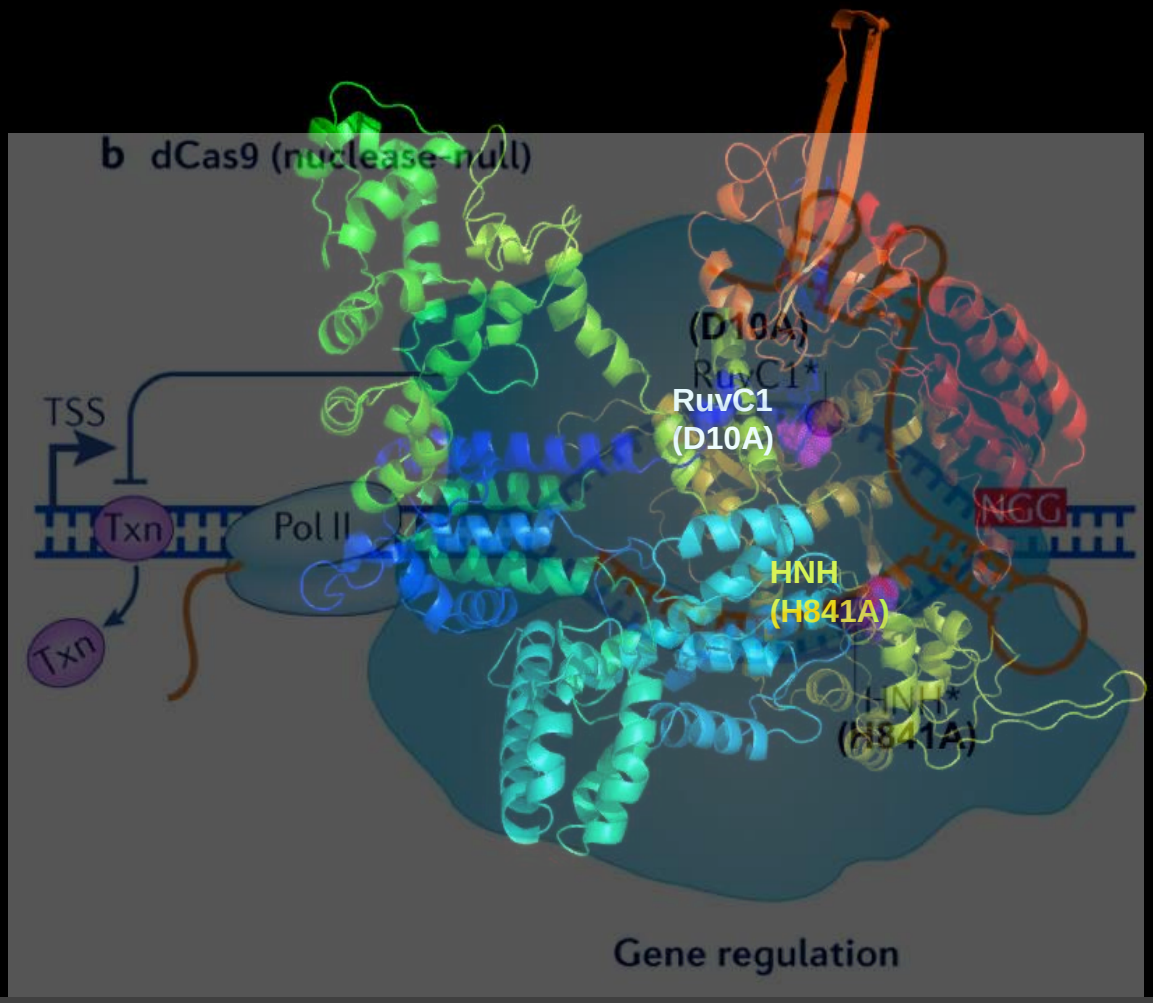


Cas9 consists of a nuclease (NUC) lobe and a recognition (REC) lobe



From
Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation
Antonia A. Dominguez, Wendell A. Lim & Lei S. Qi
Nature Reviews Molecular Cell Biology **17**, 5–15 (2016) | doi:10.1038/nrm.2015.2

Cas9 consists of a nuclease (NUC) lobe and a recognition (REC) lobe



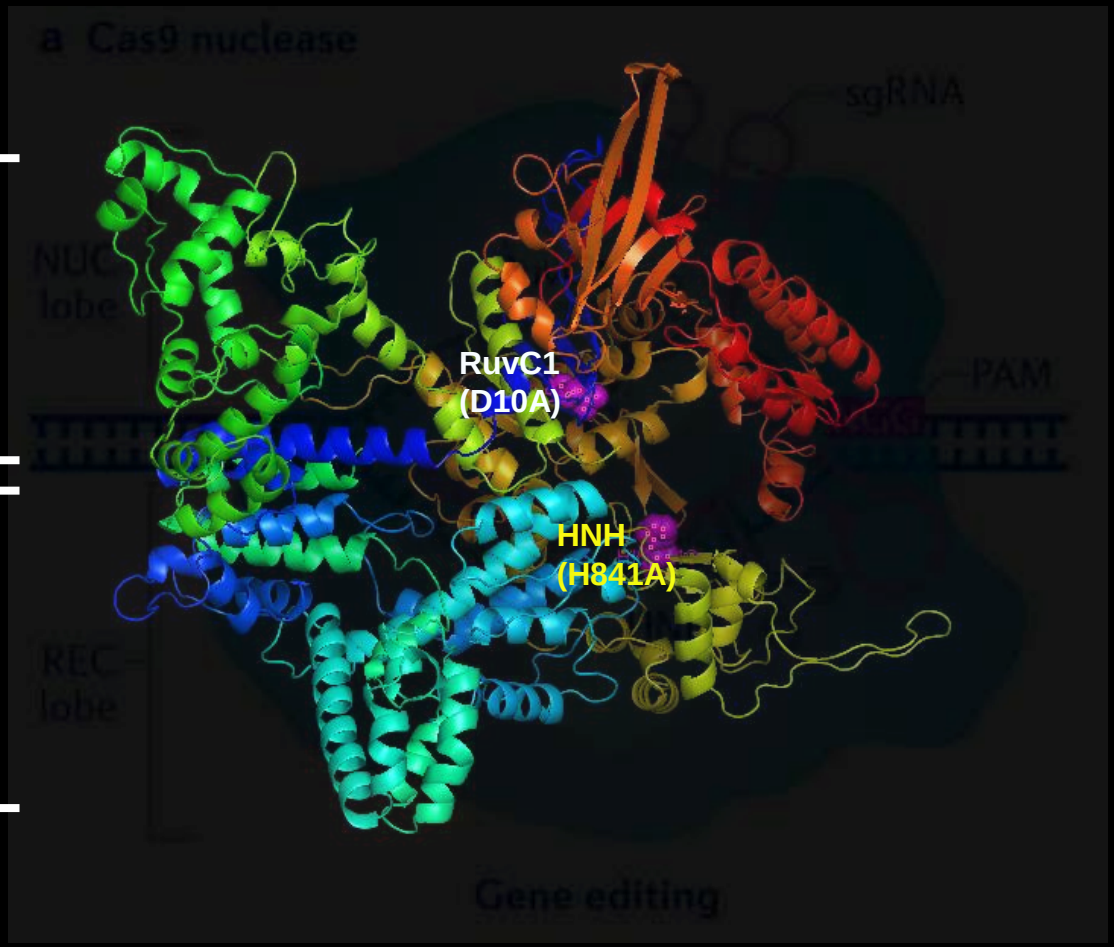
NUC lobe

REC lobe

Cas9 consists of a nuclease (NUC) lobe and a recognition (REC) lobe

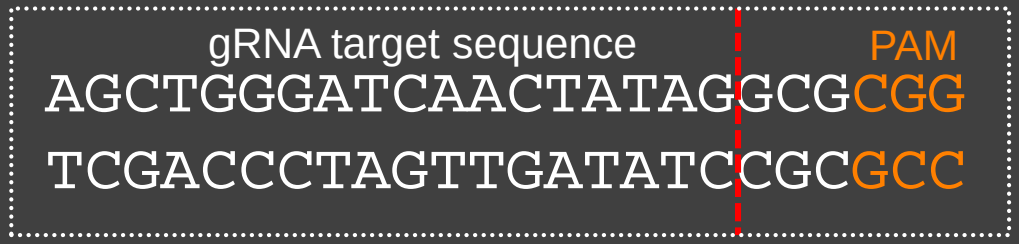
NUC lobe

REC lobe

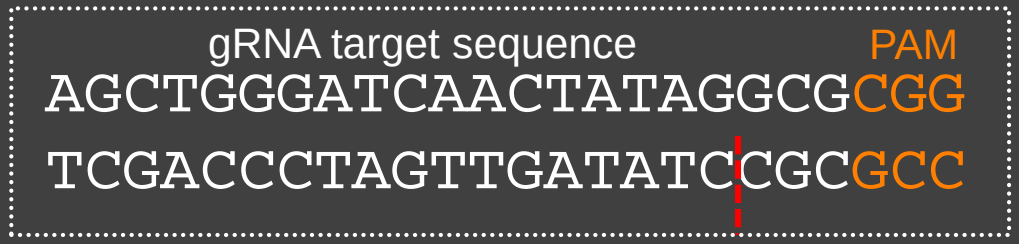


Designing a guide RNA

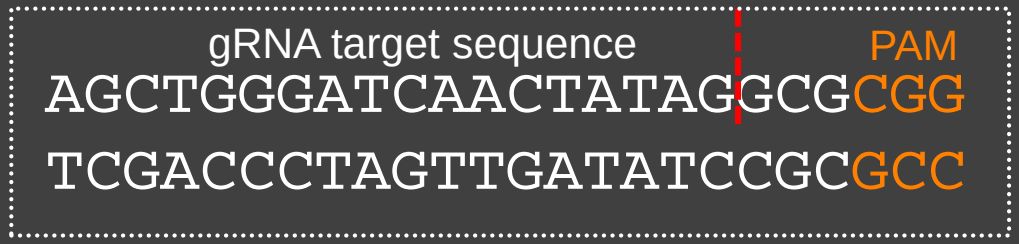
- Cas9 wild-type: The cut site occurs 3 bp 5’ of the PAM sequence



- Cas9n (D10A) nickase: a single strand nick occurs at the opposite strand



- Cas9 (H841A) nickase: a single strand nick occurs at the same strand



- dCas9 (D10A, H841A) nuclease-dead: binds to DNA but does not cut

Characterized PAMs for Cas9 orthologs

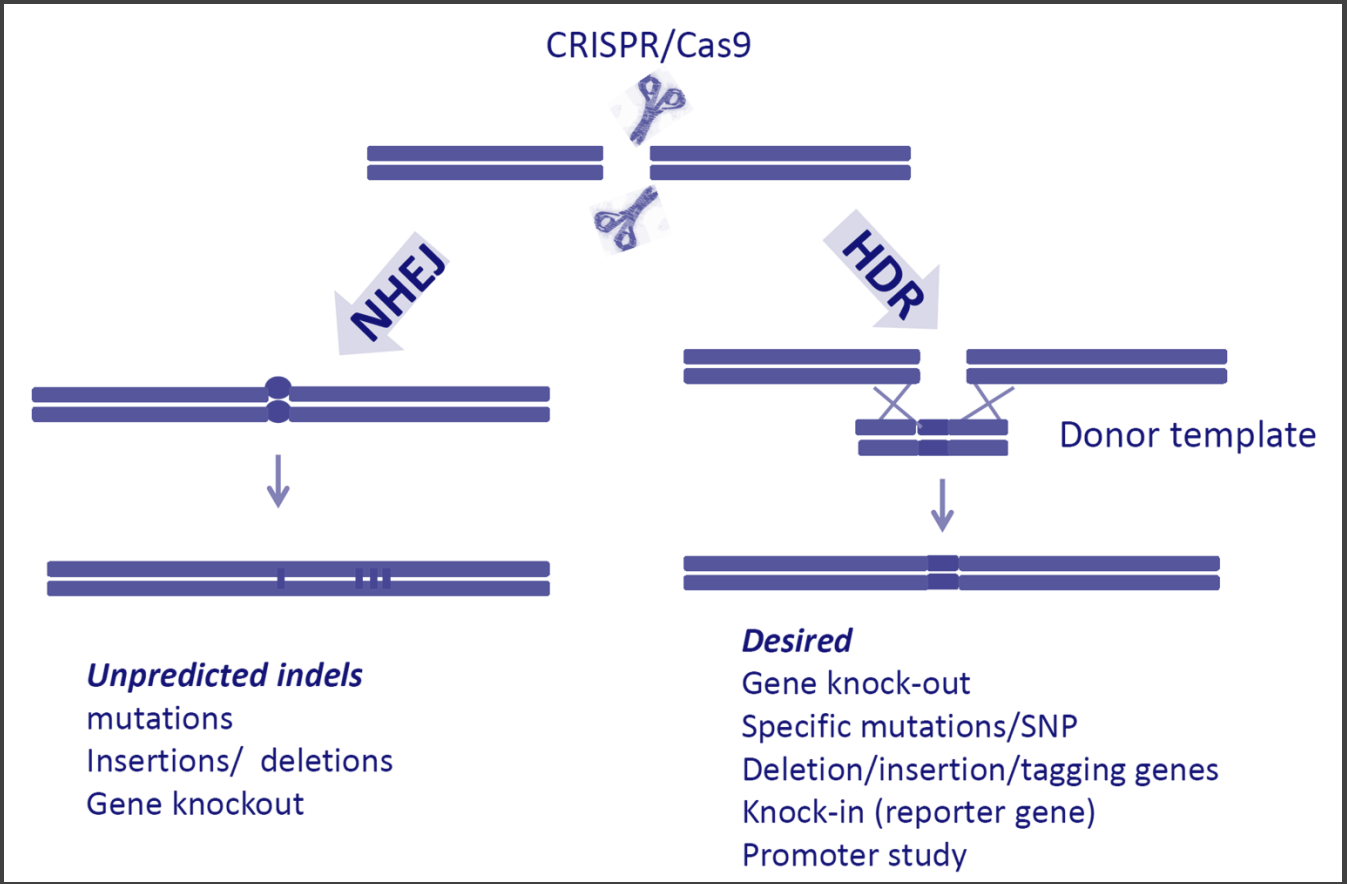
Cas9 system	PAM	References
Streptococcus thermophilus CRISPR1	NNAGAAW	Horvath et al. 2008
		Esvelt et al. 2013
Streptococcus thermophilus CRISPR3	NGGNG	Horvath et al. 2008
Streptococcus pyogenes	NGG	Mojica et al. 2009
Streptococcus agalactiae	NGG	Mojica et al. 2009
Listeria monocytogenes	NGG	Mojica et al. 2009
Streptococcus mutans	NGG	Van der Ploeg 2009
Neisseria meningitidis	NNNNGATT	Zhang et al. 2013
		Esvelt et al. 2013
Campylobacter jejuni	NNNNACA	Fonfara et al. 2013
Francisella novicida	NG	Fonfara et al. 2013
Streptococcus thermophilus LMG18311	NNGYAAA	Chen et al. 2014
Treponema denticola	NAAAAN	Esvelt et al. 2013

CRISPR/Cas9 system applications

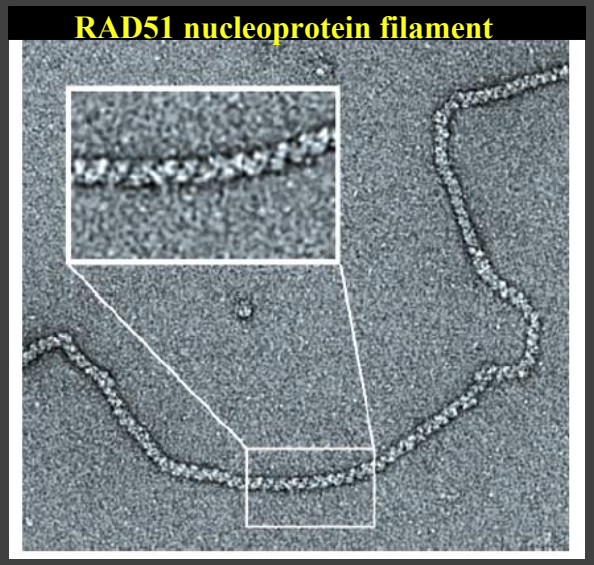
Use molecular scissor to cut genome at specific site

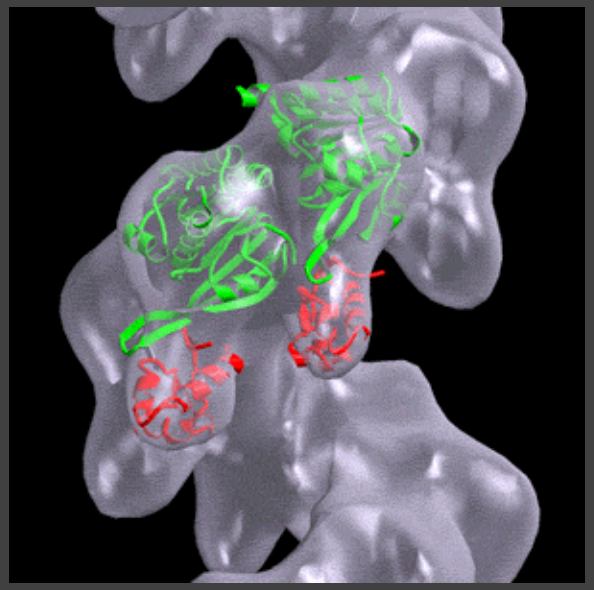
Allow the cell to repair the cut site by:

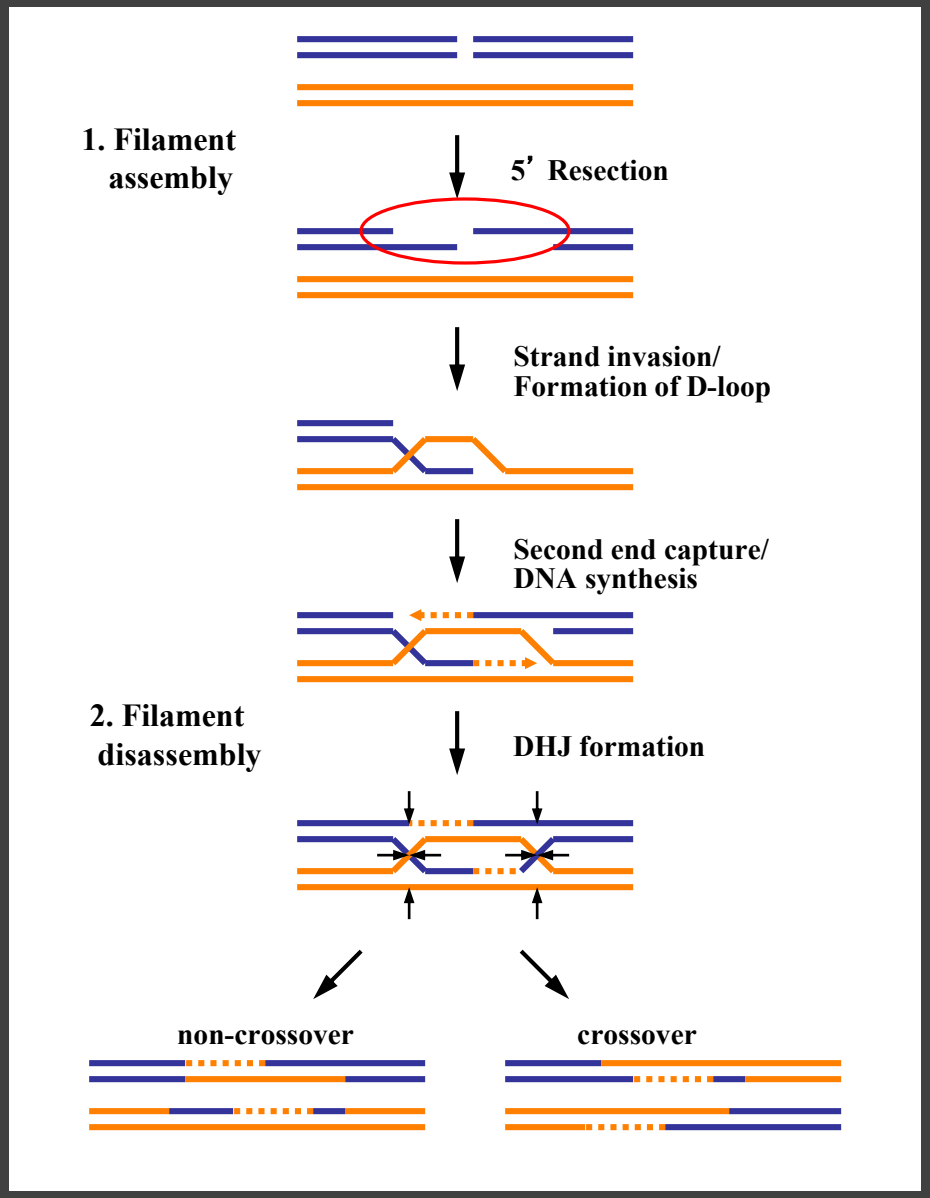
1. NHEJ DNA repair - disrupt the gene
2. HDR DNA repair - change the gene



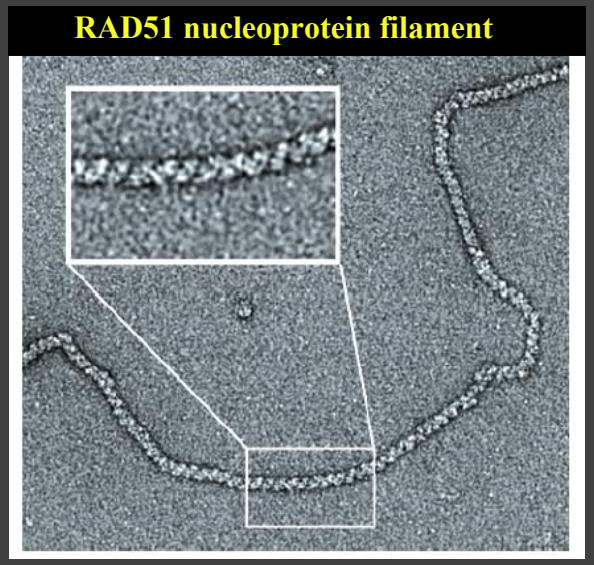
DSB Repair by Homologous Recombination

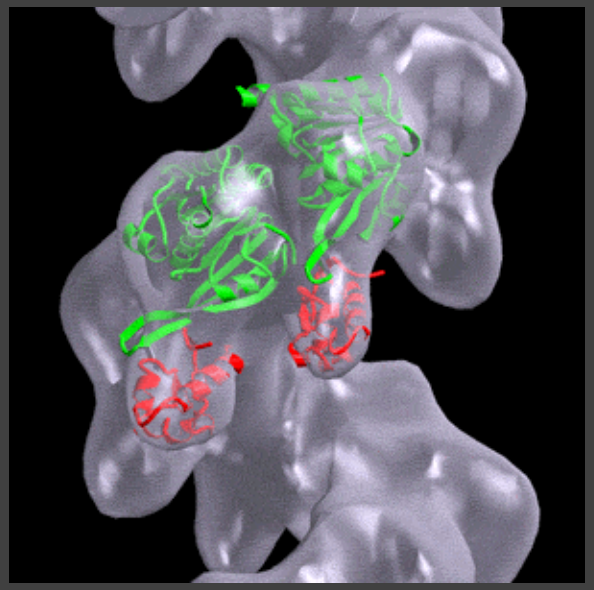


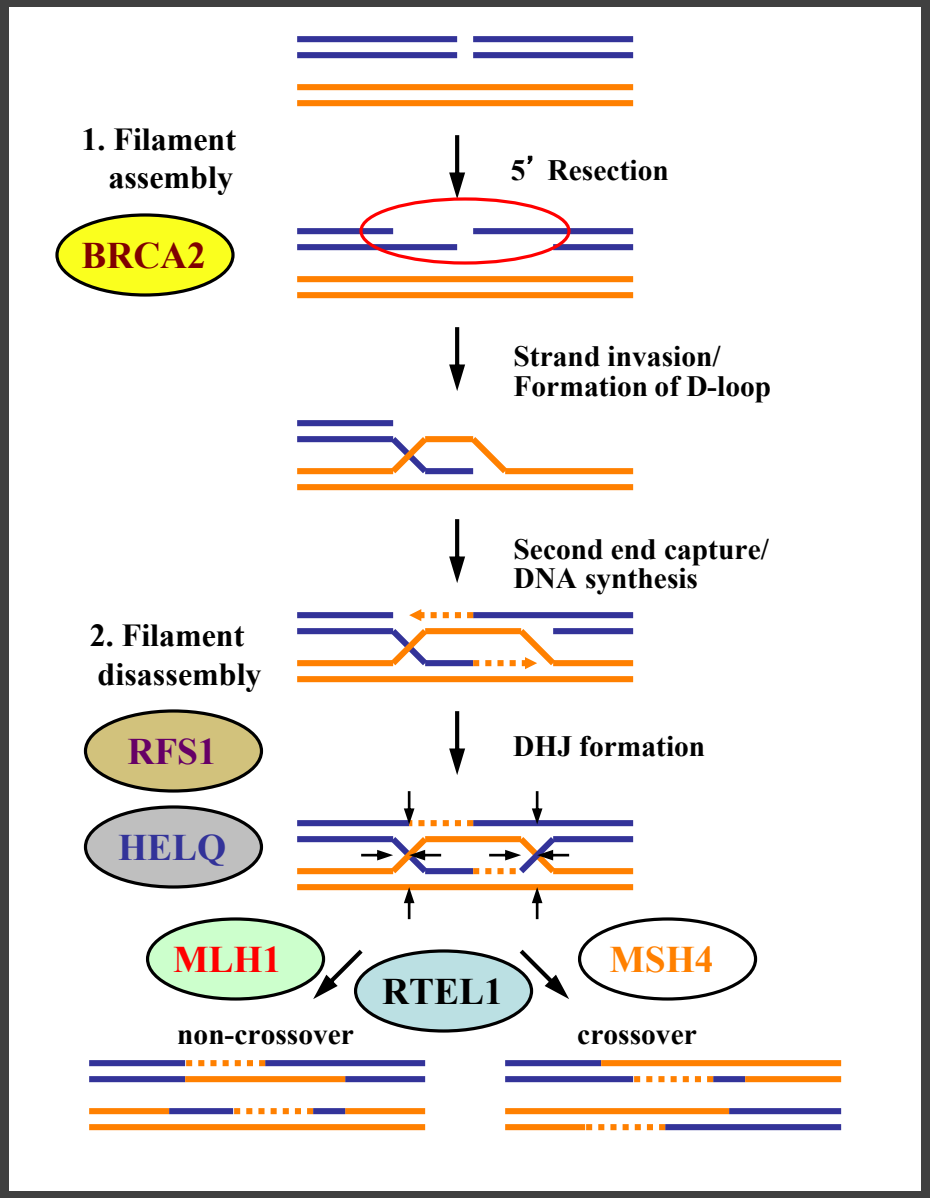




DSB Repair by Homologous Recombination





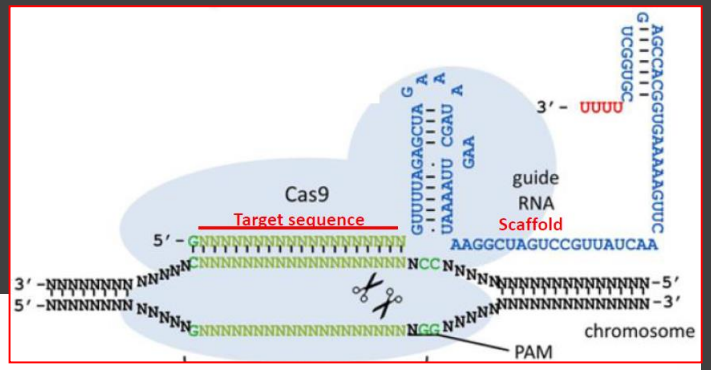


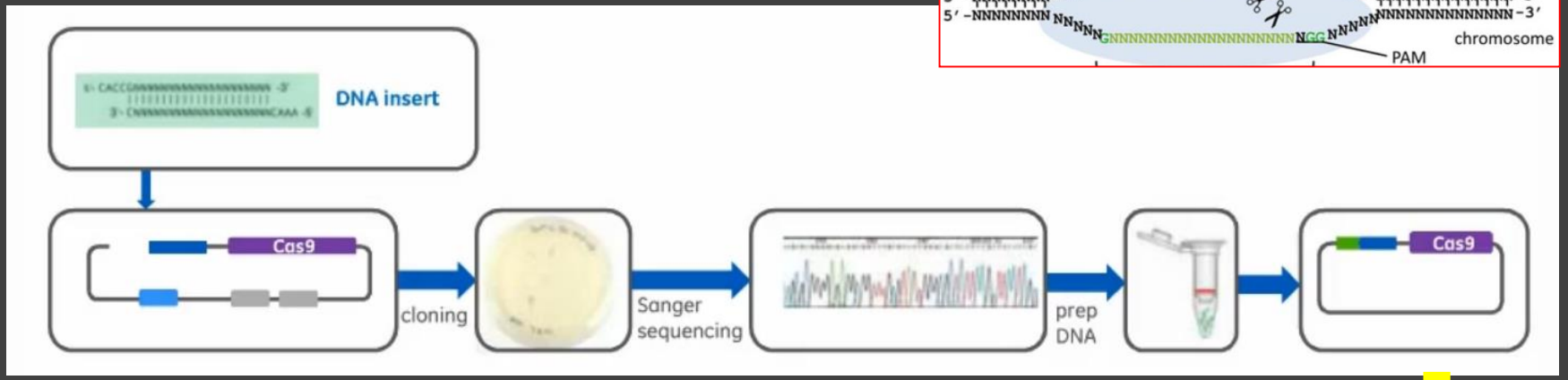
CRISPR/Cas9 general applications

- Gene disruption (without donor DNA template)
- Gene knockout (with a reporter knockin)
- Gene disruption (non-protein coding)
- Specific mutations (SNP introduction, correction, insertion, deletion, tagging endogenous gene)
- Promoter study
- Conditional knockout
- Large chromosomal deletions
- Exogenous gene insertion
- CRISPR interference (CRISPRi) and activation (CRISPRa)
- High throughput screen (Lentiviral sgRNA libraries+Cas9)

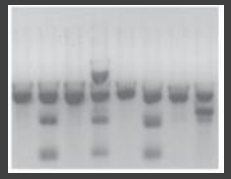
Summary of experimental workflow

1. Design and selection of targeting sequences (by algorithm)
2. Synthesis of DNA insert oligos
3. Clone into CRISPR/Cas9 expression vector (from several sources)
4. Sequencing
5. Plasmids purification
6. Transfect cells
7. Selection e.g. antibiotic
8. Clonal Isolation
9. Clonal characterization with further analysis and Phenotypic assay

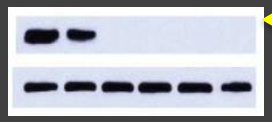




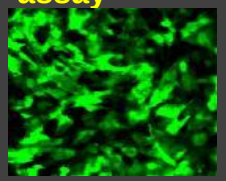
Mismatch detection assay



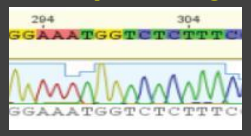
Western blot



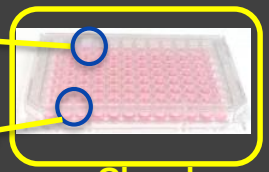
Phenotypic assay



Sanger sequencing

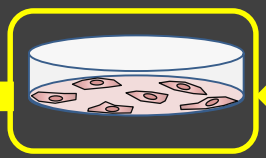


Clonal characterization

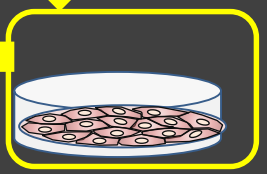


Clonal Isolation

antibiotic



antibiotic



transfection

CRISPR/Cas9 genome editing appears very simple

- identifying a gRNA target sequence
- ordering an oligo with the target sequence
- cloning the oligo into a gRNA vector
- transfecting cells with the gRNA + Cas9

… HOWEVER …

Basic experimental design considerations

- delivery of CRISPR-associated protein 9 (Cas9) and guide RNAs (gRNAs) to the target cells
- maximizing on-target activity and specificity
- evaluation of editing results (for efficacy, specificity)

AIM: high rates of the desired genome perturbation, low rates of off-target (OT) or nonspecific effects, and a good readout of the outcome.

Key considerations for CRISPR/Cas9 genome editing

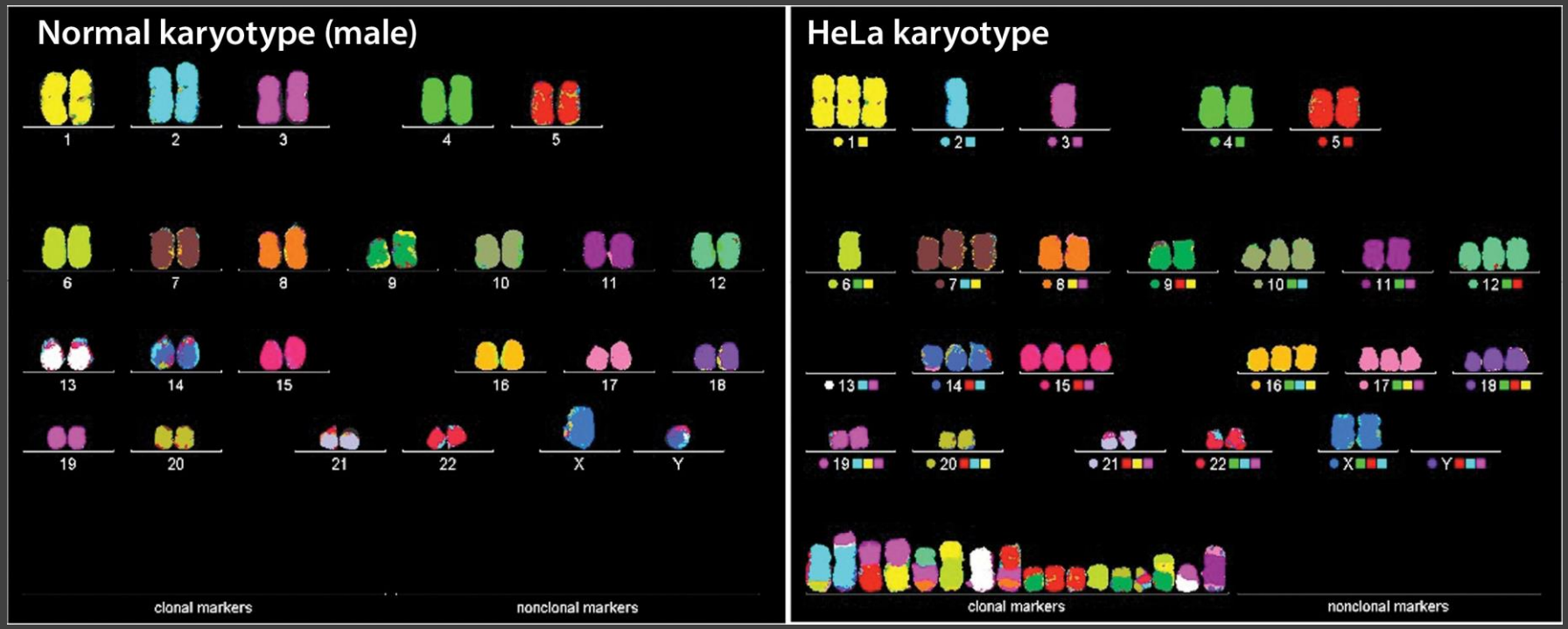
- Gene target specifics √ *How many copies?*
- Cell line √ *Is it suitable?*
- gRNA design √ *What's my goal, precision or efficiency?*
- gRNA activity √ *Does my guide cut?*
- Donor design √ *Have I minimized re-cutting?*
- Screening √ *How many clones to find positives?*
- Validation √ *Is my engineering as expected?*

While CRISPR has proven quite powerful, the editing efficiency and specificity are not perfect, thus need to optimize and validate experimental designs to achieve the best results.

Key considerations for CRISPR/Cas9 genome editing

- Gene target specifics

- ✧ gene copy number/copy number variation (CNV)
- ✧ number and nature of modified alleles
- ✧ effect of modification on growth



The karyotype of a HeLa cell is very different from the karyotype of a normal human, with extra copies of some chromosomes and missing copies of others. credit: Duesberg lab, UC Berkeley

Key considerations for CRISPR/Cas9 genome editing

- Gene target specifics

Criteria and tools for selecting sgRNAs (predicting sites favoring high activity and specificity):

On-target efficacy - *Sp*Cas9 variant (VRER) recognizes NGCG PAM sites and was reported to exhibit greater on-target specificity than wild-type *Sp*Cas9

Staphylococcus aureus Cas9 recognizes NNGRR PAM sites and exhibit greater on-target specificity compared with *Sp*Cas9, while being 1 kb smaller

best results are expected for target sites in the 5′ end of coding regions in order to produce early frame shifts and stop codons

inordinately high or low affinities of sgRNA– target-DNA duplexes negatively impact Cas9 cleavage efficiency, so chose intermediate GC content

Key considerations for CRISPR/Cas9 genome editing

- Gene target specifics

For target selection, points to consider in designing your experiments:

1. Does target gene express multiple transcripts? If yes, design sgRNA such that the exon you are targeting is present in every transcript

2. SNPs present in target site? If yes, the protospacer element of sgRNA may have difficulties binding to it. So, check first reliable database (NCBI database SNPs and UCSC Genome Browser) to ensure that the target has no SNPs

3. What is the ploidy of cells? Existence of multiple alleles lead to increase in number of possible editing events, DNA sequencing can identify type of editing events

4. What is known phenotype associated with target gene? It is important to attribute results in lethality, proliferation or differentiation to correct causal factors

5. Do you select for monoclonal population? Select as soon as possible after editing experiment, as non-edited cells could potentially outgrow edited cells

Key considerations for CRISPR/Cas9 genome editing

- Cell line
  - transfection/electroporation/microinjection/viral transduction
  - single cell dilution
  - optimal growth conditions

- Following transfection of CRISPR reagents, cells will need to be single cell diluted to obtain a clonal population.

- If a cell line tolerates being single cell diluted then plating 96-well plates at 1 cell per well in standard cell culture media is appropriate.

- If the cell line does not tolerate single cell dilution in standard media, then the use of conditioned media can often improve clone recovery.

- If the use of conditioned media in 96 well plates does not improve the recovery of clones, cells can be plated to large tissue culture dishes and individual colonies picked.

In hard-to-transfect cells, including many primary cell types, transduction with a viral vector provides an alternative, using, e.g. lentiCRISPRv2.

Whether employing transfection or transduction, Cas9 expression varies from cell to cell, and the levels also vary among cell lines.

Key considerations for CRISPR/Cas9 genome editing

- Cell line
  - transfection/electroporation/microinjection/viral transduction
  - single cell dilution
  - optimal growth conditions

Time required to achieve gene edits appears to depend on many factors:

- ✓ target gene
- ✓ cell type
- ✓ KO versus KI
- ✓ the levels of Cas9 and sgRNA

Generally, when feasible, it is necessary to wait a week or more following the introduction of Cas9 and sgRNA in order to accumulate edits in the targeted cells

Key considerations for CRISPR/Cas9 genome editing

- gRNA design

  - Sequence source
  - Off target potential
  - Guide proximity
  - Wild-type Cas9 or mutant nickase or dCas9

- After 12 bases proximal to the PAM, Cas9 can tolerate mismatches, bind and cleave non-exact target sequences.

- Optimize targeting and reduce off-target possibilities using a number of databases (E-Crisp, Off-spotter, and CRISPdirect)

- Drastically reduce off-target effects by using Cas9 nickases (Ran et al. 2013)

- Use engineered Cas9 with photocaged lysine, protein is inactive until stimulated with UV light (Hemphill et al. 2015)

Design and selection of targeting sequences (by algorithm)

Tool	Type of CRISPR/ Cas system	Sequence input	Support for Cas9 nickase	Comparison of multiple sequences	Off-target analysis	Scoring	Species support	Batch mode	Software type
ZiFiT	Type II only	Sequence only	**Yes**	No	No	No	N.A.	No	web
OptimizedCRISPR Design	Type II only	Sequence only	**Yes**	No	**Yes**	Off-target scoring	15	**Yes**	web
CRISPR Direct	Type II Only	Sequence/ Identifiers	No	No	**Yes**	Off-target scoring	18	No	web
Cas9 OnlineDesigner	Type II only	Sequenceonly	**Yes**	No	**Yes**	No	20	No	web
CHOPCHOP	Different Type II	Sequence/ Identifiers	No	No	**Yes**	Off-target scoring	19	No	web
E-CRISP	Different Type II	Sequence/ Identifiers	**Yes**	No	**Yes**	Off-target scoring	21	No	web
sgRNAcas9	Type II only	Sequence only	**Yes**	No	**Yes**	Off-target scoring	N.A.	**Yes**	local
sgRNA Designer	Type IIonly	Sequence/ Identifiers	No	No	No	**ActivityScore —type II**	N.A.	**Yes**	Web/local
CRISPRseek	Different Type II	Sequence only	**Yes**	**Yes**	**Yes**	Off-target scoring	N.A.	**Yes**	Bioc*
CRISPR MultiTargeter	Multiple types	Sequence/ Identifiers	**Yes**	**Yes**	No	**ActivityScore —type II**	12	**Yes**	web

*Bioc—Bioconductor package of the R programming and statistical environment

ZiFiT (http://zifit.partners.org/ZiFiT/)
Optimized CRISPR Design (http://crispr.mit.edu)
CRISPR Direct tool (http://crispr.dbcls.jp)
Cas9 Online Designer (http://cas9.wicp.net)
CHOPCHOP (http://chopchop.rc.fas.harvard.edu)
E-CRISP (http://www.e-crisp.org/E-CRISP/)
sgRNA Designer (http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design)
FlyCRISP Optimal target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/)
Cas-OFFinder (http://www.rgenome.net/cas-offinder/)
GT-Scan (http://gt-scan.braembl.org.au/gt-scan/)

Design and selection of targeting sequences (by algorithm)

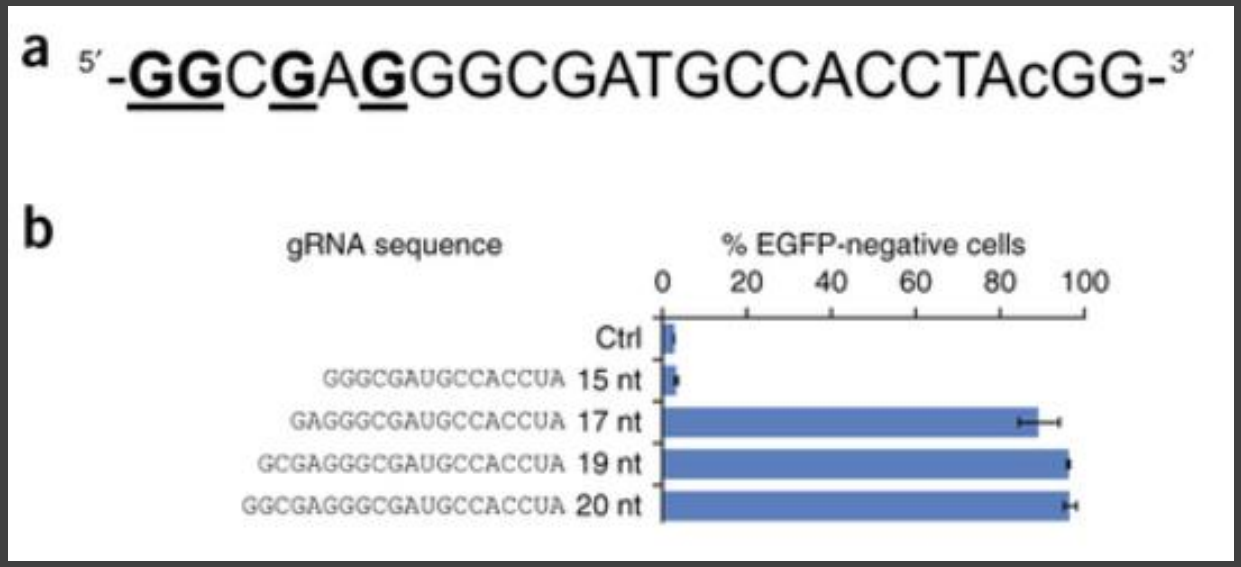
In general, more than one sgRNA is employed for each target gene, and hence multiple designs are required.

This compensates for the fact that not all sgRNAs are effective, even with the best efficacy-prediction algorithms.

Key considerations for CRISPR/Cas9 genome editing

- gRNA activity

  - Number of gRNAs
  - gRNA activity measurement



Improving CRISPR-Cas nuclease specificity using truncated guide RNAs

Yanfang Fu, Jeffry D Sander, Deepak Reyon, Vincent M Cascio & J Keith Joung

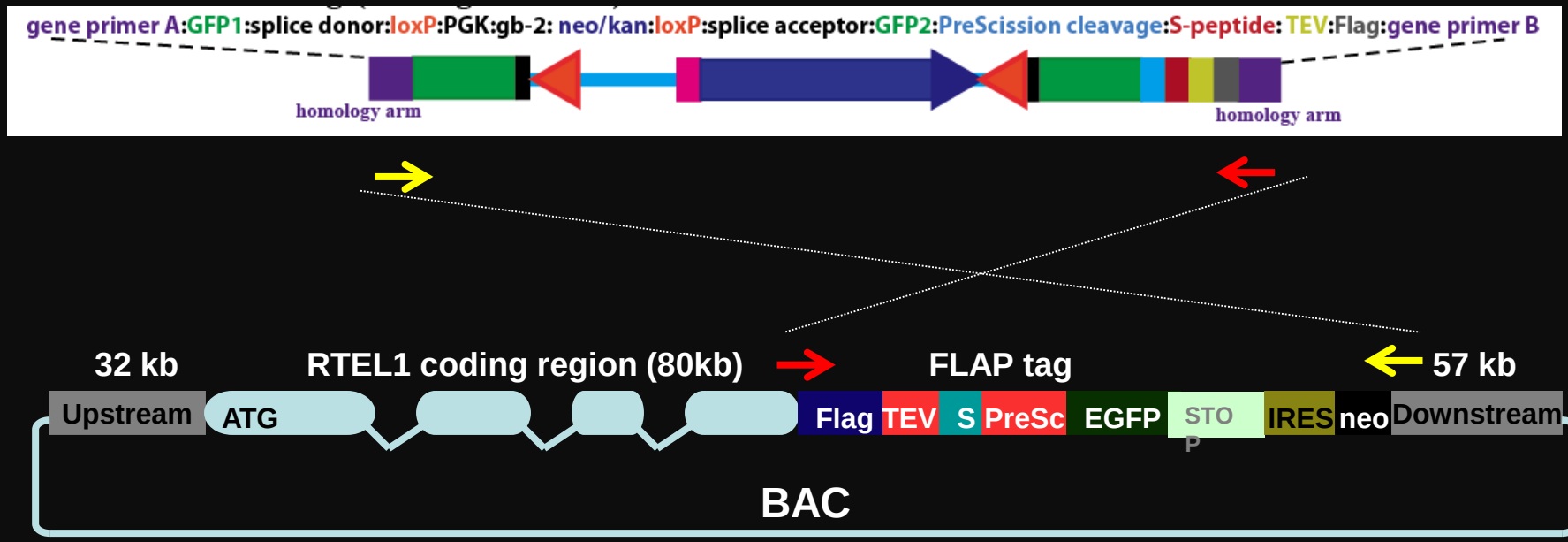
Nature Biotechnology **32**, 279–284 (2014) | doi:10.1038/nbt.2808

Received 17 November 2013 | Accepted 06 January 2014 | Published online 26 January 2014 | Corrected online 29 January 2014

Key considerations for CRISPR/Cas9 genome editing

- Donor design
- ✧ Donor sequence modification
- ✧ Modification effects on expression or splicing
- ✧ Donor size
- ✧ Type of donor (AAV, oligo, plasmid, BACs)
- ✧ Selection based strategies

C-terminally FLAP-tagged RTEL1 (RTEL1_CFLAP) stably expressed in 3T3 cells



Science. 2013 Oct 11;342(6155):239-42. doi: 10.1126/science.1241779.

RTEL1 is a replisome-associated helicase that promotes telomere and genome-wide replication.

Vannier JB[1], Sandhu S, Petalcorin MI, Wu X, Nabi Z, Ding H, Boulton SJ.

Red/ET & CRISPR/Cas

Add the advantages of recombineering to the power of CRISPR/Cas9 to extend your genome studies

CRISPR/Cas9 permits rapid knock-outs or site directed mutagenesis but is not well suited to larger and more complicated genome engineering exercises. These can be accomplished with Red/ET Recombination (recombineering) to generate targeting constructs or BAC transgenes that compliment CRISPR/Cas9 applications.

For example, instead of using CRISPR/Cas9 in sequential steps to introduce multiple mutations in a gene, each of which may need to be introduced into both endogenous alleles and then characterised in situ, it is less work with a more flexible outcome to introduce the mutations into a BAC transgene, which is introduced over a CRISPR/Cas9 knock-out.

In many cases, it is easier to do the precise work in *E.coli* using recombineering:

- well characterized BAC libraries exist for human, mouse and most model organism; annotated BAC clones available from different suppliers;

- BACs normally carry an entire genomic locus of a gene of interest including all necessary regulatory elements;

Key considerations for CRISPR/Cas9 genome editing

- Donor design

When designing KI strategies, consider the DNA break location:

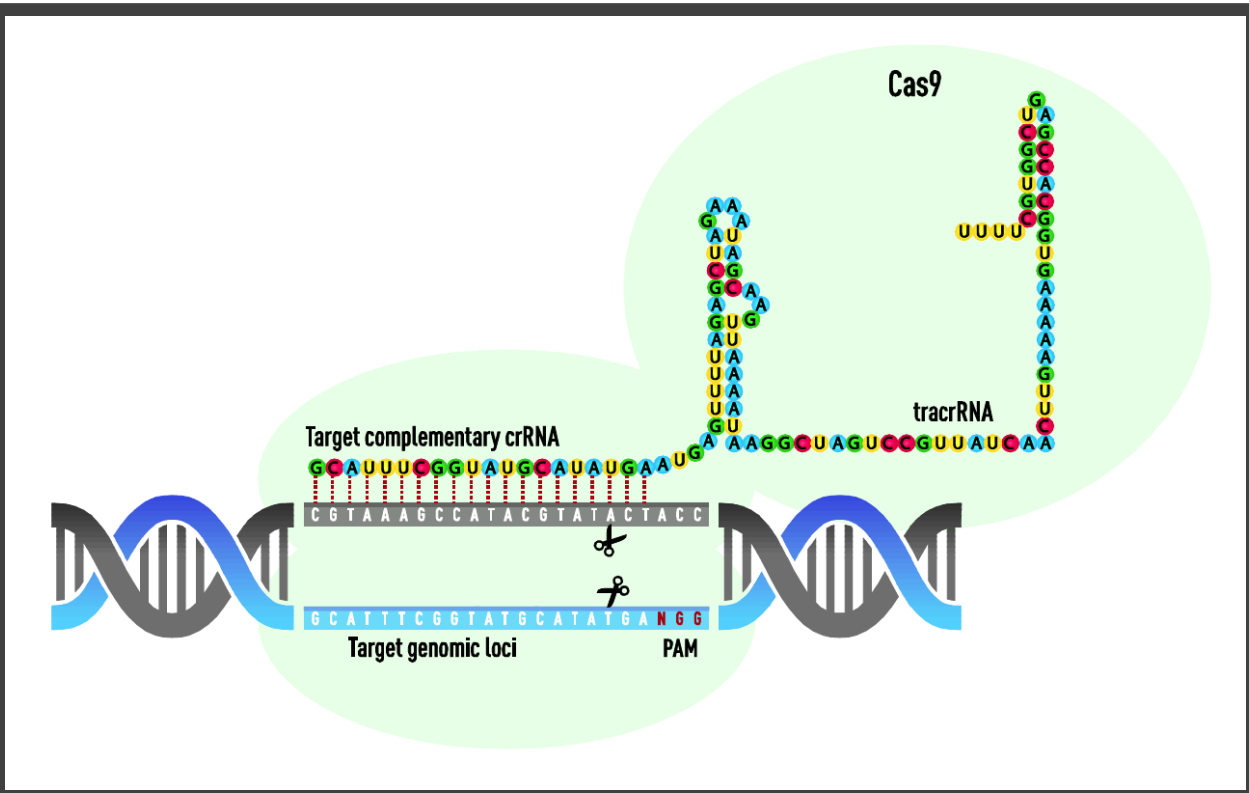
For small mutations (single-nucleotide replacements), a DSB close to site of mutation can be repaired with a ssDNA oligo with the desired mutation and about 50-nucleotide flanking homology arms

To introduce large insertions such as GFP reporters, use longer repair template such as a targeting plasmid with 400- to 1000-bp homology arms on either side of the mutation site

When using ssDNA,dsDNA plasmids or PCR products, mutate the targeted PAM site to prevent subsequent cleavage of modified or repaired alleles

Introduce several silent mutations at the sgRNA-binding site of template to create a distinct primer-binding site in repaired alleles to facilitate genotyping with a new restriction enzyme recognition site

crRNA:tracrRNA combined into sgRNA



Cleavage occurs on both strands, 3 base pairs upstream of the NGG photospacer adjacent motif (PAM) sequence on the 3’ end of the target sequence

Components of CRISPR-based gene editing:

1. the Cas nuclease Cas9 (a double-stranded DNA endonuclease)
2. a target complementary crRNA(gRNA) and an auxiliary transactivating crRNA

GeneArt CRISPR nuclease vector

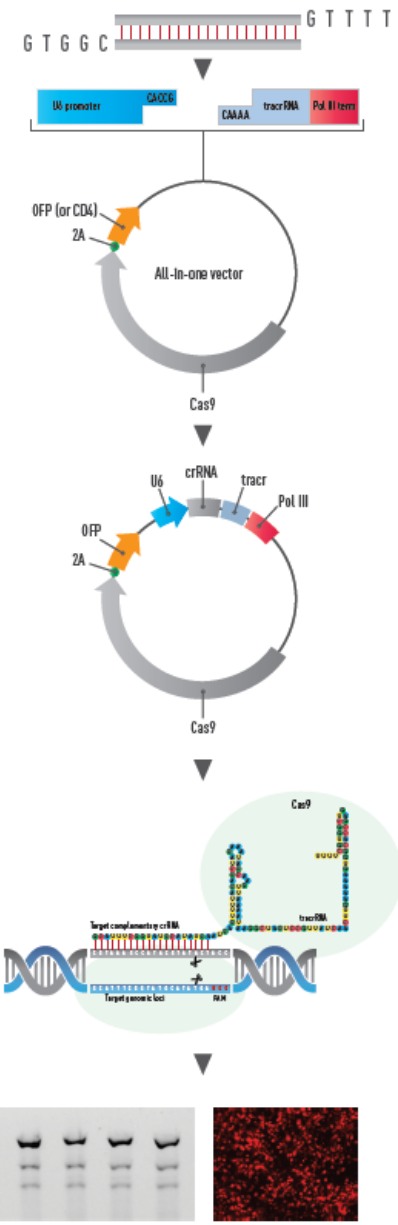
Step	Action
1	Design single-stranded DNA oligonucleotides.
2	Anneal single-stranded oligonucleotides to generate a double-stranded oligonucleotide.
3	Dilute double-stranded oligonucleotide to working concentration
4	Clone double-stranded oligonucleotide into CRISPR Nuclease Vector.
5	Transform One Shot® Chemically Competent TOP10 *E. coli* cells and select for expression clones.
6	Analyze transformants for the presence of insert by sequencing.
7	Prepare purified plasmid DNA and transfect the cell line of choice.

Anneal DNA oligos that code for target-specific crRNA

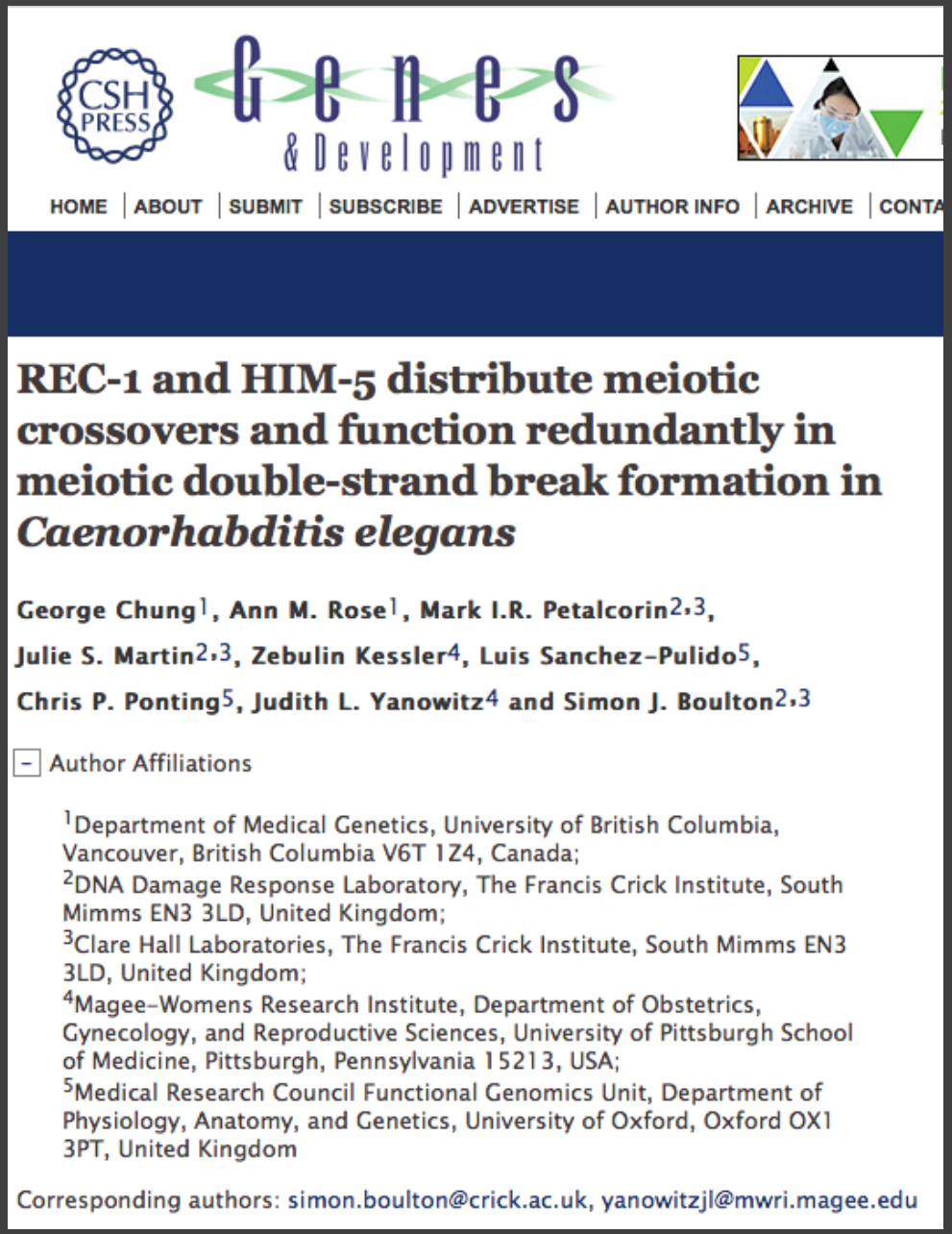
Clone annealed oligos into linearized Cas9 nuclease reporter vector using T4 DNA ligase

Transform into *E. coli* competent cells and screen for desired CRISPR clone

Transfect, enrich, and screen for gene editing



CRISPR/Cas9 system application in model organism



CSH PRESS Genes & Development

HOME | ABOUT | SUBMIT | SUBSCRIBE | ADVERTISE | AUTHOR INFO | ARCHIVE | CONTA

REC-1 and HIM-5 distribute meiotic crossovers and function redundantly in meiotic double-strand break formation in *Caenorhabditis elegans*

George Chung[1], Ann M. Rose[1], Mark I.R. Petalcorin[2,3], Julie S. Martin[2,3], Zebulin Kessler[4], Luis Sanchez-Pulido[5], Chris P. Ponting[5], Judith L. Yanowitz[4] and Simon J. Boulton[2,3]

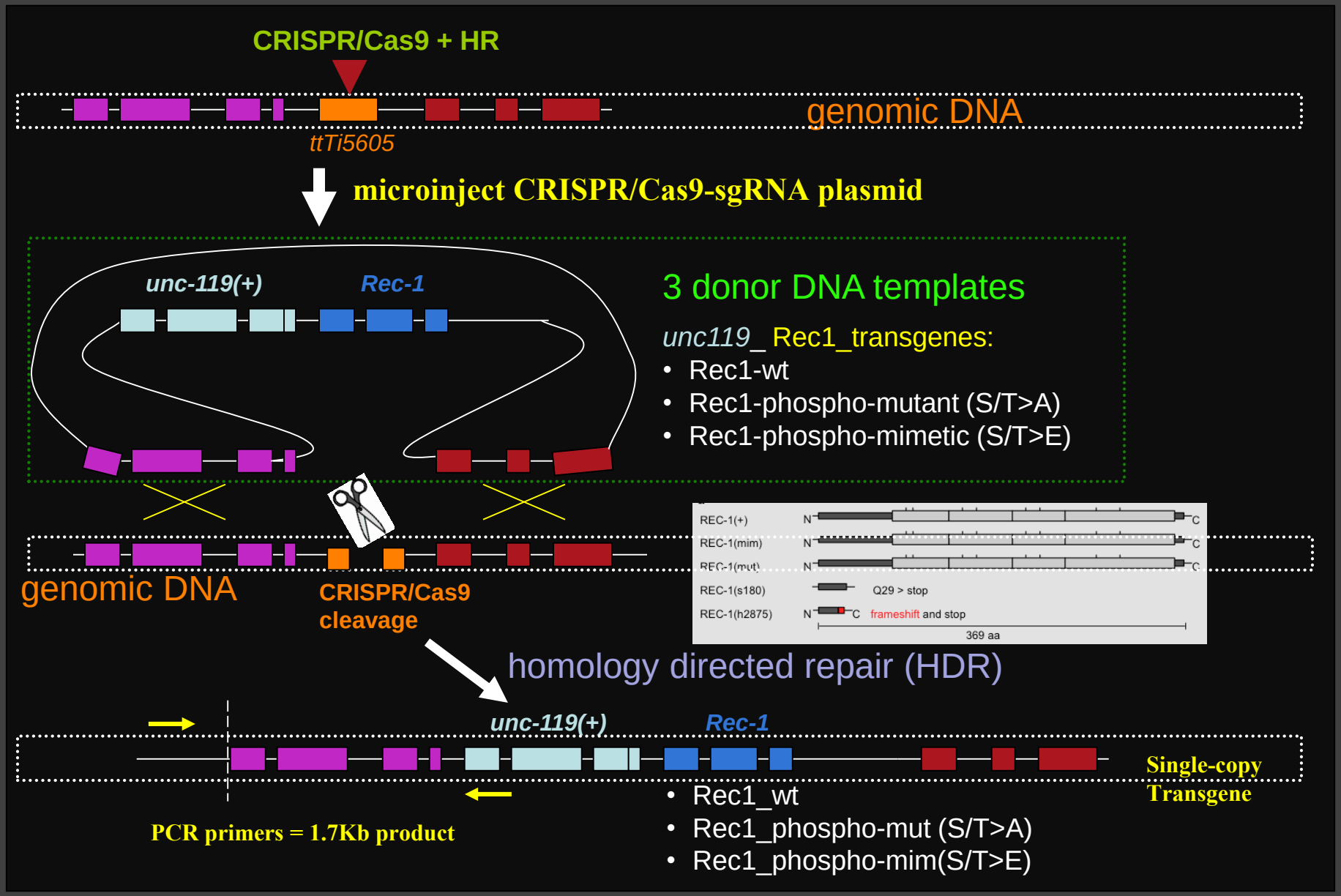
– Author Affiliations

[1]Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada;
[2]DNA Damage Response Laboratory, The Francis Crick Institute, South Mimms EN3 3LD, United Kingdom;
[3]Clare Hall Laboratories, The Francis Crick Institute, South Mimms EN3 3LD, United Kingdom;
[4]Magee-Womens Research Institute, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, USA;
[5]Medical Research Council Functional Genomics Unit, Department of Physiology, Anatomy, and Genetics, University of Oxford, Oxford OX1 3PT, United Kingdom

Corresponding authors: simon.boulton@crick.ac.uk, yanowitzjl@mwri.magee.edu

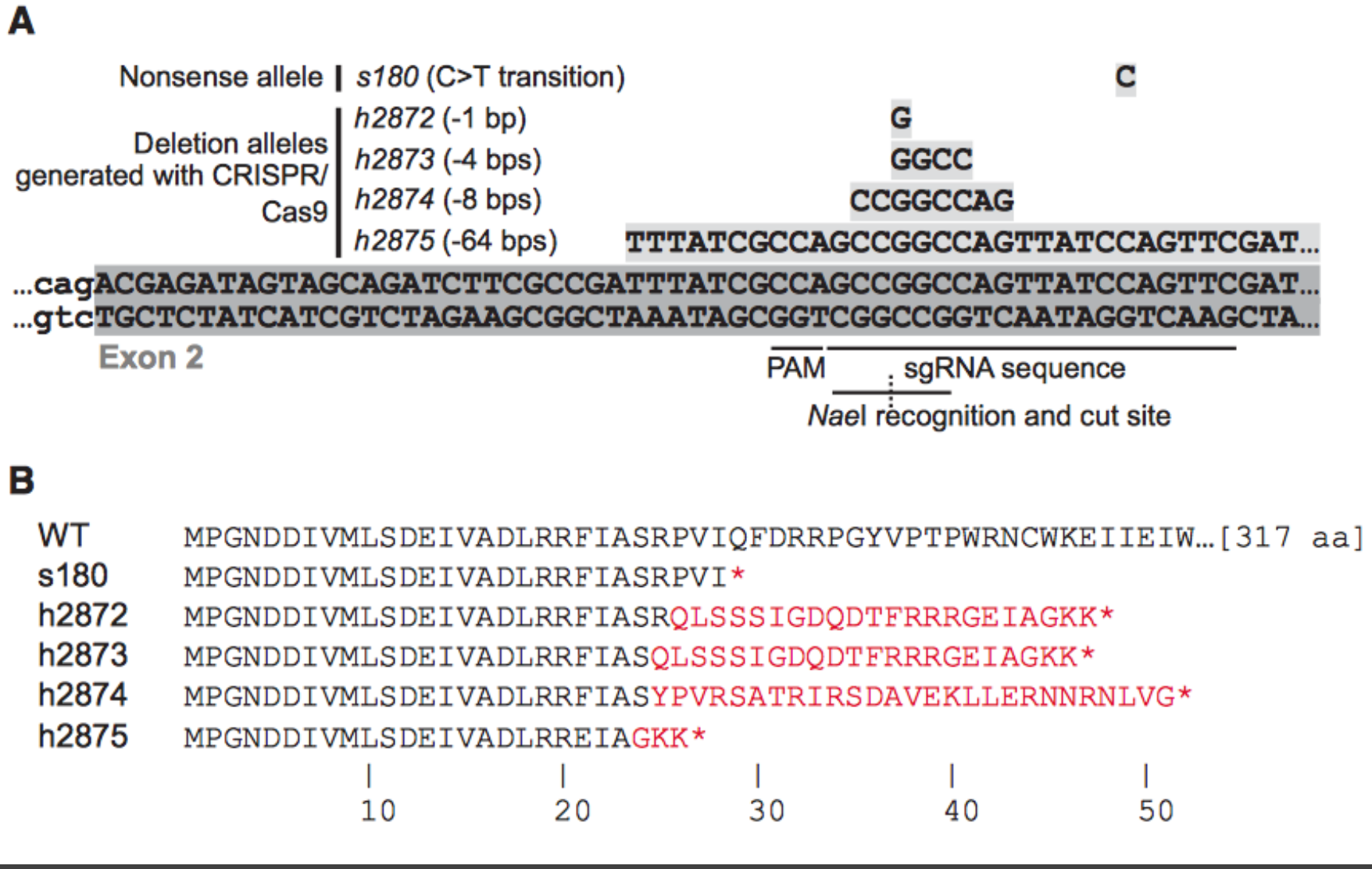
Chung, Rose, Petalcorin *et al.* (2015) GENES & DEVELOPMENT 29:1969–1979

Single insertion of Rec-1_wt rescue rec-1 phenotype



Chung, Rose, Petalcorin *et al.*(2015) GENES & DEVELOPMENT 29:1969–1979

Four alleles of rec-1 generated by CRISPR/Cas9



Chung, Rose, Petalcorin *et al.*(2015) GENES & DEVELOPMENT 29:1969–1979

Applications of CRISPR/Cas9 across industries

Therapeutics

Agriculture

Research Tools

CRISPR/Cas9 applications

Protein Production

Transgenic Animals

Acknowledgment

Universiti of Brunei Darussalam
PAPRSB Institute of Health Science

Dr Adi Idris
Inflammasome and cell death pathways

Dr Ihsan Nazurah Zulkipli
Cancer cell biology, anti-cancer effects of medicinal plants, microtubules and cell division

Dr Mark Petalcorin
Protein biochemistry.

Dr Sheikh Naeem Shafqat
Protein expression-purification and Structure-function characterization

Dr Natasha Keasberry
Nanoparticles, bioimaging, ligand and metal complex synthesis (lanthanides and transition metals)

Dr Rajan Rajabalaya
Transdermal & Vesicular drug delivery system, Nano and microparticle drug delivery systems, Natural products & Herbal, cosmetic & toiletries formulation

Dr Sheba David
Biomaterials, Pharmacological studies
Laser assisted transdermal delivery
Natural products & its screening
Vaginal & Microparticle drug delivery

Dr Shirley Lee
Investigating anti-cancer effects of local medicinal plants, neurology and ageing-associated neurodegenerative diseases.

Dr Ya Chee Lim
Kinases, phosphorylation, cancer.

Md Nuh Musa

Dr Zen Huat Lu

Comparative genomics, bioinformatics, next-generation sequencing, infectious diseases

Nurul Ramizah Hj Zulhilmi

Yee Ping Cheng

Atiqah Sulaiman

يونيرسيتي بروني دارالسلام
KE ARAH KESEMPURNAAN INSAN

Acknowledgment

DNA Damage Response Lab, Clare Hall
Simon Boulton
Jordan Ward
Julie Martin
Carrie Adelman
Kerstin Gari
Zuzana Horejsi
Visnja Pavicic-Kaltenbrunner
Jean-Baptiste Vannier
Jillian Youds
Antonia Tomas-Loba
Rafal Lolo
Martin Taylor
Jennifer Svendsen
Ross Chapman
Kenichiro Matsuzaki

C. elegans
Genetics Center
Shohei Mitani

University of the Philippines
Makati Medical Center
Francisco S. Chung Jr.

Imperial College
Enrique Martinez Perez

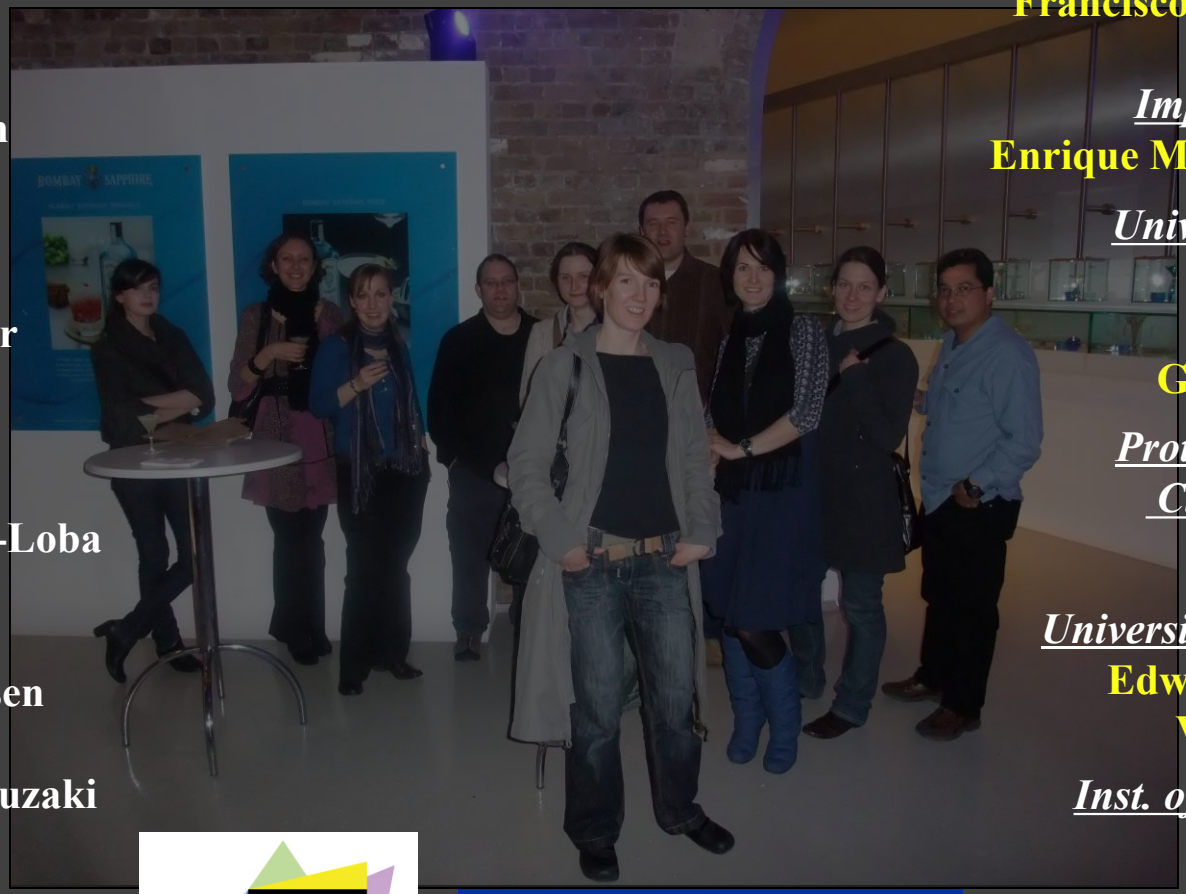
University British Columbia
Ann Rose
George Chung

Protein Analysis – Clare Hall Labs
Mark Skehel

University of Virginia
Edward Egelman
Vitold Galkin

Inst. of Cancer Res.
Dale Wigley

Harvard Medical School
Dana Farber Cancer Ins..
Alan D'Andrea
Raphael Ceccaldi



THE FRANCIS CRICK INSTITUTE

CANCER RESEARCH UK